

SOME EFFECTS OF FOREST TREE ROOTS ON MYCORRHIZAL BASIDIOMYCETES

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INTRODUCTION

A large number of Basidiomycetes are known to live in symbiosis with forest trees such as members of Pinaceae, Betulaceae and Fagaceae, forming ectotrophic or ectendotrophic mycorrhizas with them. Among the Hymenomycetes, tree mycorrhizal fungi have been demonstrated experimentally in the following genera: *Amanita*, *Boletinus*, *Boletus*, *Cantharellus*, *Clitopilus*, *Clitocybe*, *Cortinarius*, *Entoloma*, *Lactarius*, *Lepiota*, *Paxillus*, *Russula* and *Tricholoma*, by Melin (1922, 1923 *a, b*, 1924, 1925 *a, b*, 1936), Melin & Nilsson (1953), Hatch & Hatch (1933), Doak (1934), Modess (1941), Rayner & Levisohn (1941), Santos (1941), Fries (1942), Norkrans (1949), Hacskeylo (1951, 1953), Hacskeylo & Palmer (1955), Bryan & Zak (1961) Vozzo & Hacskeylo (1961) and others. In addition, there are probably several other genera, for instance, *Gomphidius*, *Hebeloma*, *Hydnum*, *Hygrophorus* and *Inocybe* which contain mycorrhizal fungi. Among the Gasteromycetes only *Rhizopogon* and *Scleroderma* are known to contain mycorrhiza-forming species. Whether Ascomycetes such as *Elaphomyces* and *Tuber* form mycorrhizas with forest trees remains to be established (Melin, 1959 *a*). *Cenococcum graniforme*, the most unspecific of all known mycorrhizal fungi, is supposed to be the imperfect stage of an Ascomycete (Lihnell, 1942).

It is remarkable that many different Basidiomycetes are able to form mycorrhizas with the same tree species. More than forty species have been proved to form mycorrhiza with *Pinus silvestris*, which has been studied most thoroughly in this respect. The actual number may be many times larger. Even a single tree may be associated with many fungal species at one time.

On present evidence the mycorrhizal Basidiomycetes may be considered as root-inhabiting fungi, in the sense of Garrett (1950, 1956); the hyphae enter the rootlets and, under certain conditions, induce the characteristic mycorrhizal structures. According to this view the fungal symbionts may be primarily parasites which obtain from the host certain metabolites essential for their development. When, however, the

symbiotic state of equilibrium is established, the higher partner generally also benefits from its fungal associate. As confirmed lately (see Harley, 1959; Lobanow, 1960), tree mycorrhizas act as nutrient-absorbing organs which, in most habitats, are more efficient than the uninfected roots.

Our knowledge of the mechanisms leading to the formation of normal mycorrhizas is still rather limited and we have therefore made extensive studies in this Institute in the last few years to obtain a better understanding of these fungus-root relationships. In the first instance we have attempted to throw some light upon host effects on the fungus which may be important for the establishment of symbiotic relations, and this aspect of ectotrophic mycorrhizal associations forms the main subject of this paper.

TRANSLOCATION OF CARBON-CONTAINING MATERIALS FROM THE HIGHER PLANT TO THE FUNGAL ASSOCIATE

By means of the isotope technique, Uppsala workers have recently proved that the basidiomycete associate, under pure culture conditions, obtains considerable amounts of carbon-containing materials from its higher partner (Melin & Nilsson, 1957). Pine seedlings were cultured aseptically in Erlenmeyer flasks and inoculated with mycelia of either *Boletus variegatus* or *Rhizopogon roseolus*. When mycorrhizas had formed, the intact seedlings were exposed for 0.5–1 hr. to an atmosphere containing C^{14} -labelled CO_2 , at c. 70 % of daylight in clear weather. It was clearly demonstrated that carbon-containing compounds formed in photosynthesis are transported in considerable amounts from the root to the fungal associate. A similar flow of organic materials from the mycotrophic plant to its fungal associate may occur in nature, although it may vary quantitatively as well as qualitatively with the external and internal conditions. Among the transported substances there are no doubt some essential to the mycorrhizal fungi and their association with the root.

This finding of Melin & Nilsson is in line with observations of several workers that roots of many higher plants give off many substances when immersed in distilled water, or different solutions. Some root secretions have been identified as amino acids (Virtanen & von Hausen, 1951; Ratner, 1954; Linskens & Knapp, 1955; Rovira, 1956; and others), nucleic acid constituents (Lundegårdh & Stenlid, 1944; Stenlid, 1947; Fries & Forsman, 1951), B-group vitamins (West, 1939; Rovira & Harris, 1961), and different kinds of enzymes (Koupreyitch, 1954;

Ratner, 1954). Roots of mycotrophic plants such as pine have also been found, in this Institute and by Slankis (1958), to release vitamins and amino acids. According to Slankis (1958) they also exude sugars (glucose and arabinose) to some extent.

CARBON SUPPLIES

There is evidence that the mycorrhizal fungi, in their symbiosis with trees, obtain substances from the roots that serve as carbon and energy sources. Several workers (Melin, 1923*a*, 1936; Hatch & Hatch, 1933; Modess, 1941; Norkrans, 1949, and others) have shown that mycorrhizas are readily formed by several Basidiomycetes in purified sand in the presence of mineral nutrients and very small amounts of 'starter' glucose. No doubt the roots were the source of carbon and energy for the symbiotic fungus in these experiments as well as in other experiments of longer duration (Melin, 1925*b*).

Frank (1885) suggested that the tree mycorrhizal fungi obtain carbohydrates from their host. Björkman (1942, 1944, 1949), Harley & Waid (1955), and others (see Harley, 1959) have produced evidence supporting this view.

Physiological investigations on tree mycorrhizal Basidiomycetes have indicated that many prefer simple carbohydrates as carbon and energy sources (Melin, 1925*b*, 1953; How, 1940; Norkrans, 1950, and others). Generally they cannot utilize cellulose, or have only a slight capacity for producing adaptive enzymes for cellulose decomposition. However, there are exceptions to this rule. In a few cases litter-decomposing species such as *Boletus subtomentosus* and *Tricholoma fumosum* were able to form mycorrhizas with pine under pure culture conditions (Lindeberg, 1948; Modess, 1941; Norkrans, 1950). Thus tree mycorrhizal Basidiomycetes differ widely in their behaviour towards complex polysaccharides, even though the majority seem to utilize mainly simple carbohydrates.

It seems unlikely that mycorrhizal Basidiomycetes of this latter group are capable of satisfying their requirements for carbon-containing material from the humus of the forest soils in competition with saprophytic soil micro-organisms, since the humus contains only small amounts of soluble carbohydrates. In nature, tree roots may be the main carbon and energy source of these fungi. Cellulose-decomposing basidiomycete associates, on the other hand, may get their carbon requirements from the soil as well as from the roots. When they are in mycorrhizal association with trees, they probably do not produce

cellulase, so long as soluble carbohydrates are available from the roots. However, when the roots no longer provide an excess of such substances, they utilize cellulose (Norkrans, 1950) which they may find in the roots as well as in the soil (Melin, 1962*a*).

Even though the carbohydrates in the host may be essential to 'sugar-requiring' basidiomycete symbionts, as is the case with many obligate parasites (Allen, 1954), this cannot explain all associations of mycorrhizal Basidiomycetes with tree roots. Neither can it explain the characteristic development of the fungal associates in the rootlets, or the balanced status of the mycorrhizas. This point of view accords with the observations of MacDougal & Dufrenoy (1944, 1946) that new mycorrhizas of normal appearance were formed continuously on detached segments of pine roots which had remained alive for several seasons, indicating that carbon-containing materials suitable for the particular fungal symbiont occurred in the soil used in these experiments. Apparently the mycorrhizal roots, in this case, obtained their carbon supplies from the soil through the fungus.

Harley (1959) also expressed the opinion that free sugars in the host root are not a factor leading to the production of ectotrophic mycorrhizas. Handley & Sanders (1962) suggested that in the search for factors controlling the formation of A- and B-type ectotrophic mycorrhizal associations, factors other than soluble reducing substances in the root should not be neglected.

SUPPLIES OF ESSENTIAL VITAMINS AND AMINO ACIDS

Tree mycorrhizal Basidiomycetes studied by Uppsala workers (Melin & Lindeberg, 1939; Melin & Norkrans, 1942; Melin & Nyman, 1940, 1941; Melin, 1953, and unpublished; Norkrans, 1950) were generally heterotrophic for one or more B-vitamins, when grown in synthetic nutrient medium in pure culture. All those tested were partially or completely dependent on thiamine or its constituent moieties, pyrimidine and/or thiazole (Melin, 1954). Most proved to be relatively thiamine-heterotrophic, and only a few were totally so. The degree of heterotrophy varied widely for different species and to some extent even within the same species (Melin & Nyman, 1941). Chudjakow & Wozniakowskaja (1951) have reported thiamine autotrophic strains of tree mycorrhizal Basidiomycetes, and Rawald (1962) reported such strains in *Tricholoma imbricatum* and *T. pessundatum*, but Swedish strains of these two species studied by Norkrans (1950) required thiamine. Some mycorrhizal

Basidiomycetes have one or more additional vitamin requirements *in vitro*. *Tricholoma imbricatum* is relatively heterotrophic for pantothenic acid (Norkrans, 1950), and *T. fumosum* and *Lactarius deliciosus* are partially heterotrophic for nicotinamide (Norkrans, 1950; Melin, 1953).

Table 1. *Effects of L-glutamic acid (100 μ mol./20 ml. medium) and α -ketoglutaric acid (100 μ mol.) on the growth rate of various strains of three pine mycorrhizal Basidiomycetes in buffered ammonium medium*

(In each case the total nitrogen was initially 3 mg./flask (20 ml. medium). Initial pH 5.6.)

	Incubation period (days)	Ammonium phosphate (mg. N/flask)	Glutamic acid (mg. N/flask)	α -Keto- glutaric acid (μ mol./flask)	Dry weight (mg.)	Final pH
<i>Boletus variegatus C</i>	12	3.0	—	—	8.0	5.4
		1.6	1.4	—	21.4	5.6
		3.0	—	100	10.6	5.4
<i>B. variegatus E</i>	10	3.0	—	—	7.8	5.2
		1.6	1.4	—	18.7	5.3
		3.0	—	100	14.7	5.3
<i>B. variegatus H</i>	10	3.0	—	—	13.0	4.7
		1.6	1.4	—	22.8	4.9
		3.0	—	100	16.5	4.9
<i>B. luteus E</i>	7	3.0	—	—	12.1	5.1
		1.6	1.4	—	17.6	5.3
		3.0	—	100	13.4	5.2
<i>B. luteus F</i>	14	3.0	—	—	4.8	5.3
		1.6	1.4	—	24.6	5.2
		3.0	—	100	8.2	5.4
<i>B. luteus G</i>	7	3.0	—	—	8.7	5.3
		1.6	1.4	—	15.3	5.4
		3.0	—	100	8.8	5.4
<i>Rhizopogon roseolus B</i>	7	3.0	—	—	11.0	5.3
		1.6	1.4	—	22.9	5.3
		3.0	—	100	16.7	5.3

The majority of tree mycorrhizal Basidiomycetes studied were stimulated *in vitro* by one or more amino acids, particularly glutamic and aspartic acid, or their corresponding keto acids, in the presence of ammonium nitrogen (Norkrans, 1950, 1953; Melin, 1953). However, different species and even different strains have different demands (Melin, 1955, and unpublished). Most species responded positively to glutamic acid but the dose-response curves varied, probably due mainly to different sensitivity to its toxic action. Several species such as *Boletus luteus*, *B. variegatus* and *Rhizopogon roseolus*, reached their optimal growth at levels of 100–500 μ mol./20 ml. medium. Among species most sensitive to the inhibitory action of glutamic acid were *B. versipellis* and *Lactarius rufus*, which showed positive growth response only at concentrations up to c. 1 μ mol./20 ml. Above this concentration there was

marked growth inhibition. Great differences in the response of various tree mycorrhizal Basidiomycetes to aspartic and some other amino acids were also observed.

In nature, tree mycorrhizal Basidiomycetes may obtain essential B-group vitamins and amino acids (or corresponding keto acids) from their hosts as well as from the forest soil (Melin, 1953), but the different response of various fungal associates to these metabolites seems to indicate that this does not offer an explanation for the formation of ectotrophic or ectendotrophic mycorrhizas.

EFFECTS OF OTHER ROOT METABOLITES

In numerous earlier experiments of Melin and his collaborators, many basidiomycete species—several suspected of forming mycorrhiza with trees—did not grow, or developed only very poorly in pure culture; the same experience has frequently been encountered elsewhere. The failure of growth was thought to be due, at least partly, to deficiencies of some complex substances occurring in the higher partner (Melin, 1953). I therefore decided to study the effects of living pine roots on the growth of different types of tree mycorrhizal Basidiomycetes in nutrient solutions (Melin, 1954, 1959*a*, 1962).

(a) *Qualitative experiments in 'maximum' nutrient medium*

In the first group of experiments, aseptic pine roots obtained from root cultures or seedlings grown *in vitro* were tested. Later, growth effects of root exudates of pine seedlings grown in pot cultures were also studied.

(i) *Preliminary experiments.* Aseptic roots of *Pinus silvestris* were cultured in Erlenmeyer flasks according to the method of Slankis (1951) for about 5 months. Their dry weight was then 10–15 mg. The nutrient solution of these root cultures was then replaced by a medium found in this Institute to be especially favourable for the growth of many mycorrhizal Basidiomycetes. The basic solution was generally supplemented with a mixture of B-group vitamins and amino acids (Melin & Das, 1954). For convenience, this will be called the maximum nutrient solution. As inocula, mycelial suspensions (Wikén, Keller, Schelling & Stöckli, 1951) were generally superior to floating mycelia (Melin & Lindeberg, 1939; Norkrans, 1950).

The effects of pine roots on the growth rate of fourteen fungal species are illustrated in Table 2. Although all the responses were strongly positive, growth stimulation varied considerably for different fungi. The

weakest response occurred in *Boletus subtomentosus*, the strongest in slow-growing species, such as *Russula xerampelina*, *Pholiota caperata* and *Cortinarius glaucopus*. In *R. xerampelina* practically no growth occurred in the control series in 38 days, whereas with addition of the roots there was an average mycelial yield of 25 mg. dry weight. It is noteworthy that the mycelia in this case developed almost entirely around the root, as illustrated in Pl. 1, fig. 1.

Table 2. *Growth-promoting effect of cultured roots of Pinus silvestris on various mycorrhizal Basidiomycetes added as mycelial suspensions to nutrient solution supplemented with 19 amino acids and 10 vitamins*

Fungus	Days of incubation	(Initial pH 5.1.)			
		Control		Added pine roots	
		Dry weight (mg.)	Final pH	Dry weight (mg.)	Final pH
<i>Amanita muscaria</i> (L. ex Fr.)	22	22.5	3.6	51.7	3.2
<i>A. pantherina</i> (DC. ex Fr.)	21	8.8	4.3	32.7	3.3
<i>Boletus bovinus</i> L. ex Fr.	13	14.4	3.8	34.2	3.5
<i>B. edulis</i> Bull. ex Fr.	14	5.9	4.9	23.0	4.0
<i>B. Grevillei</i> Klotzsch, E	13	14.9	4.7	43.7	3.3
<i>B. luteus</i> L. ex Fr., A*	10	33.0	3.4	69.5	3.2
<i>B. subtomentosus</i> Sw. ex Fr.	11	23.2	3.3	35.3	3.3
<i>B. variegatus</i> Sw. ex Fr., D	10	12.2	4.0	62.4	3.9
<i>Cortinarius glaucopus</i> (Schaeff. ex Fr.) Fr.	21	1.4	5.0	19.0	4.2
<i>C. multififormis</i> Fr.	14	4.7	5.0	12.2	4.4
<i>Lactarius deliciosus</i> (L. ex Fr.) Fr.	21	5.0	4.6	13.0	4.1
<i>Pholiota caperata</i> Pers. ex Fr.	22	1.2	5.0	21.4	4.3
<i>Rhizopogon roseolus</i> (Corda) Th. Fr., B	10	11.8	4.2	74.0	3.3
<i>Russula xerampelina</i> Schaeff. ex Fr.	38	0.1	4.9	24.9	3.4

* Inoculated as floating mycelia.

The pH of the culture media generally fell considerably during the experiment, although much more rapidly with, than without added roots. There was no correlation between these pH changes and the effect of roots on fungal growth.

It was assumed from these and similar experiments that the pine roots produce—besides vitamins and amino acids—one or more growth-promoting metabolites called factor M, which are essential to the growth of the fungal symbionts. *Russula xerampelina* could apparently utilize the nutrient solution only in the presence of this factor, indicating that it was totally, or almost totally heterotrophic for the M-factor. In most other fungi the M-factor increased the rate of growth but did not appreciably affect the maximum mycelial yields. This indicates that these

species were themselves capable of synthesizing the M-factor but not as rapidly as needed for optimal growth. They may therefore be considered as partially heterotrophic for this factor.

The M-factor is not specific for pine roots and is also produced by the roots of many other species (Melin, 1954; Melin & Das, 1954). As tomato roots grow easily in pure culture, they were used to work out methods for further studies of root effects on mycorrhizal Basidiomycetes.

(ii) *Diffusible and non-diffusible M-factor*. The M-factor seems to be composed of at least two components (Melin, 1955, 1959a, 1962). This was suggested by the following observation.

When the above-described experiment was discontinued, some rootlets which were surrounded by mycelium and sometimes somewhat swollen (Slankis, 1948), were fixed and embedded according to Jackson (1947). L. Orrhage (unpublished) found that hyphae of the mycorrhizal fungi had generally entered the cortex of these rootlets, mainly intercellularly but also intracellularly, although no mycorrhizal structures were observed. This entry into the rootlets may indicate that they contained not only diffusible but also non-diffusible material, essential to the fungi in a maximum nutrient solution.

Table 3. *Effects of cultured primary pine roots and their exudate on the growth-rate of Boletus variegatus D in maximum nutrient solution at 25°*

(Incubation period 6 days. Initial pH 5.2. Average of three observations with standard errors.)

Treatment	Dry weight of mycelium (mg.)	Final pH
A. Control	8.2 ± 1.5	4.2
B. Exudate (diffused out from one root in 6 days)	15.4 ± 1.2	3.8
C. Root after 6 days of exudation	24.5 ± 1.7	3.5
D. Root after 6 days of exudation and extraction at 100°	21.5 ± 2.5	3.6
E. Fresh specimen of cultured root	27.6 ± 2.0	3.5

Further evidence for this assumption was obtained from comparison of the growth of *Boletus variegatus* with added root exudates, and in the presence of roots with different pre-treatments (Table 3). Six-month-old cultured pine roots were placed in maximum nutrient solution for 6 days (treatment B), and then transferred to new flasks with the same medium. Half the roots were transferred directly (treatment C), the other half were extracted at 100° in redistilled water for 5 min. and water-

washed repeatedly before re-suspension (treatment D). In a fifth treatment, fresh specimens of cultured roots were added to maximum nutrient solution. All flasks were inoculated simultaneously with measured amounts of a hyphal suspension of *Boletus variegatus*. The exudate caused a doubling of the mycelial yield after 6 days as compared with the control (Table 3), but the roots which had given off this exudate (treatment C), and also the extracted roots (treatment D) showed a considerably higher growth-promoting activity than the exudate.

These and several similar experiments support the view that the M-factor contains at least two substances, one diffusible through plasma membranes, the other bound within the cells. The latter is also available to the mycelia, presumably as a result of their enzymic activities. Treatment B contained only diffusible M-factor exuded into the medium before the fungal suspension was introduced, whereas treatment D contained only non-diffusible M-factor, i.e. substances bound in the root cells. In treatments C and E, on the other hand, there were available to the fungus the non-diffusible as well as the diffusible M-factor, the latter being liberated from the root continuously during the experimental period for 6 days. To judge from other experiments not reported here, the total amount of diffusible M-factor released in treatment E was somewhat larger than that in treatment C.

The relative amounts of the two kinds of substances could not be determined. Judging from the growth-promoting effects in treatments B and D, the bound M-factor had a somewhat higher activity than the diffusible one. However, the two treatments were not quite comparable, as the roots in treatment D had been heated.

The greater growth-promoting effect in treatment C compared with treatment B may be mainly a result of a synergistic action of diffusible and non-diffusible M-factor. It is noteworthy that both substances were inactivated in the presence of small amounts of adenine or its derivatives (Melin, 1959*b*), and this may indicate that they are chemically related.

(iii) *Exudate of aseptic attached pine roots.* Exudates from aseptic attached pine roots were obtained through the courtesy of Dr V. Slankis, who cultured seedlings of *Pinus silvestris* in closed glass cylinders (Slankis, 1951, pp. 44–6). In his experimental arrangement the roots grew aseptically in the medium, whereas shoots developed in the free air. The solution was aseptically changed as required, and was continuously aerated. Roots of 8-month-old seedlings were allowed to exude for 5 days in redistilled water, or in fresh nutrient solution. The solution containing the exudate was added aseptically to maximum nutrient solution in varying proportions, the final level of nutrients

being adjusted to be the same in all treatments. The growth-promoting effect of the exudate on *Rhizopogon roseolus* is illustrated in Fig. 1; *Boletus luteus* responded similarly.

Experiments with attached roots of pine seedlings grown aseptically in purified sand or terralite in Erlenmeyer flasks for 1–2 years, gave similar effects to those obtained with cultured roots, and it was concluded that the diffusible M-factor was also released from attached roots.

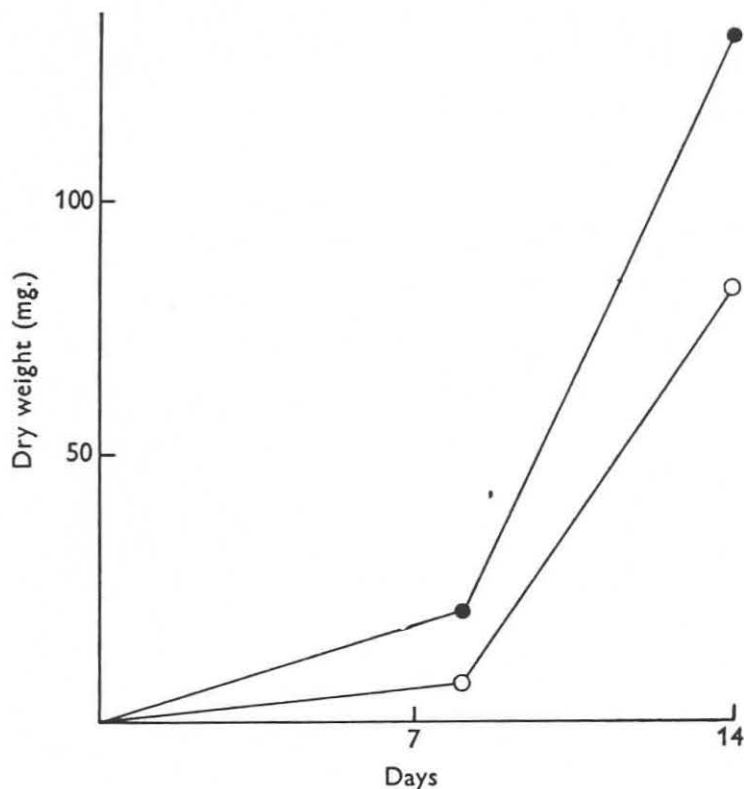


Fig. 1. Growth produced by floating inoculum of *Rhizopogon roseolus* B. in maximum nutrient solution, without supplement (hollow symbols) or supplemented with root exudate from aseptically cultured pine seedlings (solid symbols), suspended in redistilled water for 5 days.

(iv) *Auxanographic experiments.* The growth-promoting effect of the M-factor has been clearly demonstrated with several strains of *Boletus variegatus* and other mycorrhizal Basidiomycetes by means of the auxanographic method of Beijerinck (Melin, 1954, 1959a, 1962). The results of these and kindred experiments were reproducible only if washed, homogeneous mycelial suspensions were used as inocula. Measured amounts of mycelial suspensions, sieved aseptically through fine wire gauze, were thoroughly mixed with molten agar, and roots were then transferred, directly or in celluloid sacks, to the still unsolidified agar medium. If the mycelial portions had a proper size and density, species such as *B. variegatus* (for which nylon gauze with a hole size of

0.025 mm.² after repeated autoclaving and drying has been used successfully) developed strongly in the first days around the roots on the plate, resulting in a distinct auxanogram demarcated by the limit of the diffusible M-factor (Pl. 1, fig. 2).

Slowly growing species such as *Pholiota caperata*—considered more deficient for the M-factor—have also been used in auxanographic experiments (Pl. 2, figs. 3, 4). For this species, however, suspensions of relatively large mycelial fragments had to be used to get an auxanogram

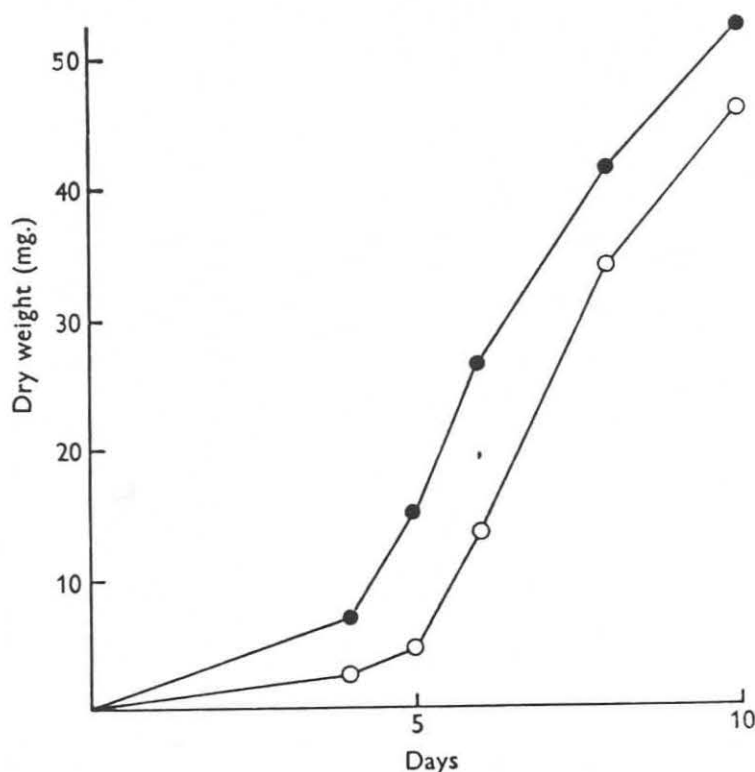


Fig. 2. Growth curves of *Boletus variegatus* (Sw.) Fr., strain K, in maximum nutrient solution without (hollow symbols) or with (solid symbols) cultured pine roots (3 months old). Inoculation with hyphal suspension.

of *Boletus variegatus* type (Pl. 2, fig. 3). Mycelial particles developed only in contact with the root (Pl. 2, fig. 4), which may indicate that they needed the synergistic action of diffusible and non-diffusible M-factor for development.

(v) *Growth response of various types of tree mycorrhizal Basidiomycetes.* The growth curves of several tree mycorrhizal Basidiomycetes were compared in maximum nutrient solution with and without cultured pine roots. Some results are illustrated in Figs. 2 and 3. Fig. 2 shows that *Boletus variegatus* responded to cultured pine roots as it did to cultured tomato roots (Melin & Das, 1954). Its growth rate was increased by the M-factor, particularly in the first stage of development, but the

maximum yield reached the same value as the control. Several other tree mycorrhizal Basidiomycetes such as *Rhizopogon roseolus*, *Lactarius rufus* (Scop. ex Fr.) Fr. and *L. mitissimus* (Fr.) Fr., produced similar growth curves.

The behaviour of three *Russula* species, illustrated in Fig. 3, was quite different. *R. fragilis* Pers. ex Fr. is a mycorrhizal symbiont with *Pinus silvestris* and *P. montana* (Melin, 1924, 1925b), and *R. sardonia*

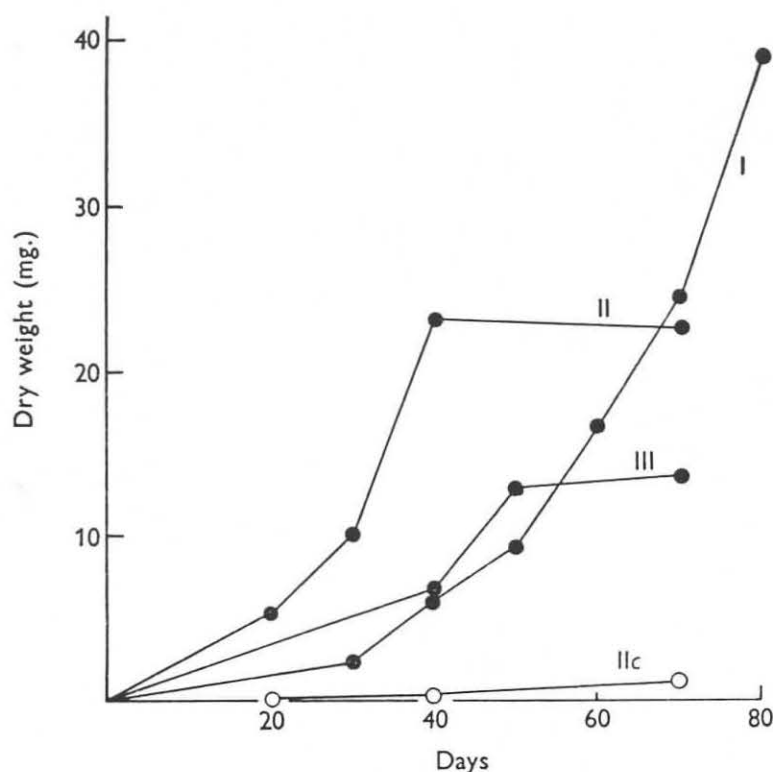


Fig. 3. Growth curves of *Russula fragilis* Pers. ex Fr. (I), *R. aeruginea* Lindbl. ap. Fr. (II) and *R. sardonia* Fr. (III) in maximum nutrient solution with supplement of cultured pine roots. Maximum mycelial yield of controls was 0.4 mg. for *R. fragilis*, 0.1 mg. for *R. sardonia*, and IIc shows the control yield for *R. aeruginea*.

and *R. aeruginea* are probably also tree mycorrhizal symbionts (Lundell & Nannfeldt, 1934, 1936; Lange, 1940). *R. fragilis* produced a growth curve (I) similar to that of *R. xerampelina* (Melin & Das, 1954). In the control series very poor growth occurred, resulting in a mycelial yield of 0.4 mg. over a period of 80 days, and no control curve is therefore given in Fig. 3. With added pine roots, however, the average mycelial yield was 40 mg., the growth rate increasing continuously during the whole incubation period after a rather long lag phase. Thus *R. fragilis* was able to utilize the nutrient solution only in the presence of the M-factor, indicating that—in contrast to *Boletus variegatus*—it was totally heterotrophic for this

factor under the experimental conditions. *Lactarius helvus* (Fr.) Fr. behaved in the same way. In *R. aeruginea* the pine root supplement also stimulated growth which, however, stopped suddenly after 40 days (Fig. 3, curve II). *R. sardonis* (Fig. 3, curve III) showed about half the growth stimulation of *R. aeruginea* and a very similar flattening of the growth curve after 50 days. These two species are therefore also totally or almost totally heterotrophic for the M-factor. At present it is not possible to say why they ceased growth after 40–50 days. One possible explanation is that they were more sensitive than *R. fragilis* to a diffusible inhibitor discussed below, which may have increased to a detrimental level in the culture flasks.

(b) *Effects of various amounts of pine root exudate*

It seemed desirable also to investigate the effect of root exudates quantitatively. Cultured pine roots as well as roots of pine seedlings grown in pots were used as test material. Amounts of exudate were measured in arbitrary units, one unit being the amount of active substance diffusing into redistilled water at 4° in 6 days from a quantity of living root equivalent to 1 mg. dry weight.

(i) *Exudate of cultured pine roots.* The response of *Boletus variegatus* to 0.3–40 units of exudate from aseptically grown roots of *Pinus silvestris* is shown in Fig. 4, curve I. Growth is expressed as percentage increase or decrease over controls (relative growth), which are represented by the horizontal line at 100. The final amount of nutrients was adjusted to be the same in all flasks. The greatest growth-promoting effect of the diffusible M-factor was obtained at levels between 5 and 10 units. At concentrations higher than 20 units the exudate exerted an inhibitory action. An amount of 40 units inhibited fungal growth by 70 % over a period of 8 days. The experiment thus confirms previous reports (Melin & Das, 1954; Melin, 1955; Melin, 1959*a*, 1962) that the exudate of cultured pine roots contains also a growth-inhibiting principle.

Two possible explanations of the inhibiting effect at higher concentrations may be suggested: (1) that cultured pine roots contain—in addition to the M-factor and other growth-promoting metabolites such as B-group vitamins—one or more growth-inhibiting substances that also diffuse from the intact root cells into the surrounding medium; (2) that the diffusible M-factor had a promoting or inhibiting effect according to its concentration.

Several observations favour the first alternative. When living primary pine roots were freeze-dried or lightly ground and placed aseptically in redistilled water, the diffusible M-factor was almost inactivated (presum-

ably by some component of the protoplast released through the injured plasma membrane), while the activity of the inhibitor increased. This is shown by comparison of curves I and III in Fig. 4. If, however, the freeze-dried or ground pine roots were autoclaved before being put in distilled water, the growth-promoting activity of the released M-factor was almost as high as for untreated roots (Fig. 4, curves I and II). It therefore appears likely that the intact pine roots also released into the surrounding medium an inhibitor which, at certain concentrations, counteracted the growth-promoting effect of the M-factor. In comparison with this the inhibitor seems to have a rather low rate of dif-

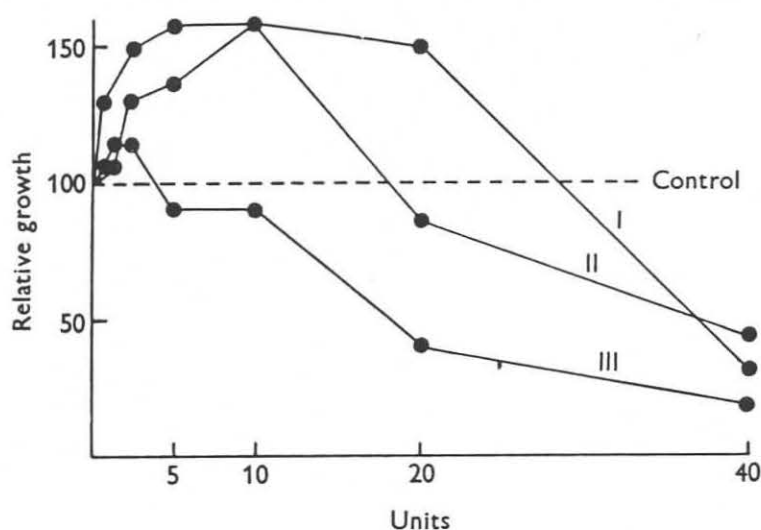


Fig. 4. Growth of *Boletus variegatus* in maximum nutrient solution supplemented with different amounts of exudate or extract from cultured pine roots treated as follows:

Curve I = exudate collected from roots shaken lightly in redistilled water for 6 days at 4°.

Curve II = extract from roots freeze-dried and then autoclaved before extraction.

Curve III = extract from roots freeze-dried before extraction.

Incubation period 8 days. Dry weight of control = 6.8 mg.

fusion through the plasma membranes of intact root cells, as previously postulated by Melin (1955). When the membranes were injured—by freeze-drying or by light grinding of the roots—the amount of inhibitor liberated was very much increased. Inhibitory effects of root exudates have been reported also from other symbiotic plants such as legumes (Nutman, 1956).

The amounts and proportions of the liberated M-factor and the inhibitor varied in different parts of the root, as was frequently demonstrated by means of the auxanographic method. The upper main axis of pine roots cultured for 6 months gave off the inhibitor in amounts sufficient to prevent completely the growth of *Boletus variegatus*, Pl. 3, fig. 5, whereas the M-factor was ineffective. On the other hand,

secondary rootlets arising from the same roots favoured fungal development (Pl. 3, fig. 6). This is in line with the finding of Lundegårdh & Stenlid (1944) that the exudation of nucleotides is much greater from parts of the root that are still growing than from somewhat older parts.

MacDougal & Dufrenoy (1944) provided some cytochemical evidence that the pine root may contain a non-diffusible inhibitor affecting the penetration of the fungal associate in the mycorrhizas. This may be true, but there is as yet insufficient experimental evidence for acceptance of the hypothesis.

(ii) *Exudates of roots of pine seedlings cultured in pots.* In 1960 seeds of *Pinus silvestris* from the same tree as in the previous series of experiments were grown in a sand-terralite mixture (1:8) in pots, and irrigated with a basic mineral nutrient solution (Melin, 1936) containing different amounts of KH_2PO_4 and $(\text{NH}_4)_2\text{HPO}_4$. The seedlings did not appear to have formed mycorrhizal associations.

Rinsed and washed roots of harvested seedlings were suspended in redistilled water as described. As the roots were inhabited by saprophytic rhizosphere microbes, particularly bacteria, the dissolved exudates had to be sterilized; parallel experiments were performed with filtered and autoclaved exudates. No attempt was made in these experiments to distinguish between exudation of intact root (and microbial) cells, and the liberation of substances resulting from breakdown and autolysis of dead root fragments, but it is thought that, as with aseptically cultured roots, the released material mainly consisted of root exudates.

Dose-response curves for *Boletus variegatus* are given in Fig. 5. It will be seen that the diffusible M-factor had its maximum activity at very low levels. Inhibition began at levels of *c.* 10 units and with 20 units the fungal growth was inhibited about 90 % over a period of 10 days. Root exudates of pot-grown pine seedlings fed with nutrient solutions containing respectively 1/20 and five times as much nitrogen and phosphorus, produced essentially similar growth-response curves, except that stimulation was greater from small amounts of autoclaved exudate from roots grown at the higher nutrient level, and both filtered and autoclaved exudates from such roots were already inhibitory at lower concentrations (*c.* 7 units). As shown in Fig. 5 exudates were always considerably more stimulatory after autoclaving than after filtration.

It is interesting to compare the results of this series of experiments with those presented in Fig. 4. In both series the exudates promoted or inhibited growth of the fungus according to their concentrations. However, the root exudates of the pot-grown seedlings became inhibitory

at much lower dosage and it is therefore assumed that they contained much larger amounts of the inhibitor, than did the exudates of cultured roots.

The simplest explanation of these differences is that they may, at least in part, be connected with a somewhat different metabolism in cultured and attached roots, and the results indicate that exudation of diffusible inhibitor from the roots varies quantitatively with internal and external conditions.

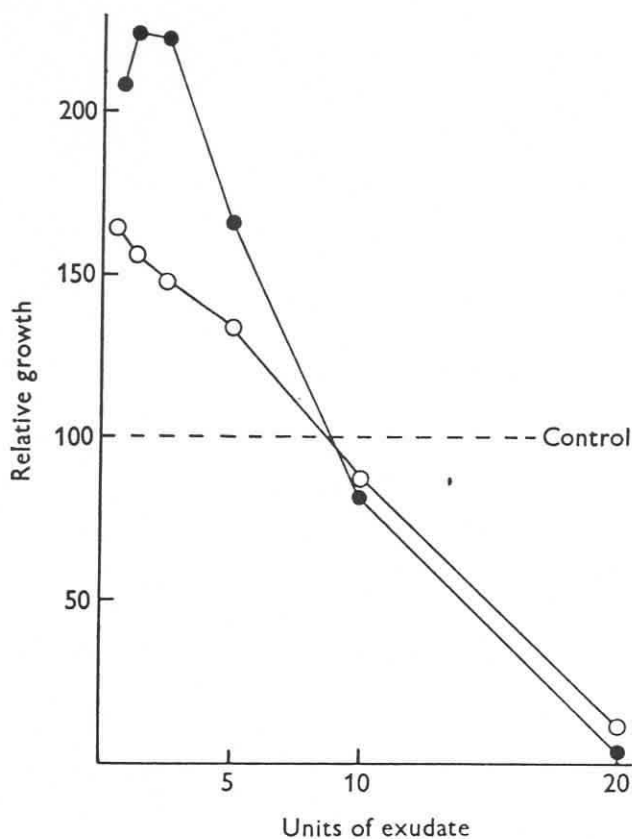


Fig. 5. Effect of different amounts of exudate from pot-grown seedlings of *Pinus silvestris* on *Boletus variegatus* F. Exudate was filtered (hollow symbols) or autoclaved (solid symbols) before adding to maximum nutrient solution. Incubation period 10 days. Dry weight of controls = 7.6 mg.

It seems surprising that the activity of the M-factor was regularly lower in the filtered than in the autoclaved exudate. A possible explanation may be a gradual inactivation of the M-factor in the filtered exudate by some heat-labile component of the protoplast, which had escaped from the root and/or the microbial cells.

There was evidence that amounts and proportions of released M-factor and inhibitor varied considerably in different parts of the root, and for the problem of mycorrhizal formation it may be of great importance to learn the behaviour in this respect of parts of the roots which are still growing.

Extensive studies have been made at this Institute to identify the substances concerned in the effects of the M-factor. H. Nilsson (1960, Report to the Swedish Council of Natural Science, unpublished), has found that the diffusible M-factor could be replaced in its effect on hyphal growth by diphosphopyridine nucleotide (DPN), which functions as coenzyme in many hydrogen transfer processes. The chemical nature of the diffusible inhibitor has not so far been identified.

CONCLUSION

The results obtained by the author and by other workers support the view that a complex of substances produced by the associated partners is involved in the formation of tree mycorrhizal symbiosis.

Some data from the present investigation suggest that excretion of growth-promoting and inhibiting root metabolites, such as the M-factor and the inhibitor, also occur under natural conditions, although the process of liberation of these substances in distilled water may not necessarily be identical with that in soil solutions. There is some indication that the exposure to distilled water may cause some injury of the plasma membranes (Laties, 1954; Fischer, 1956) and soil solutes may influence the permeability of the membranes differently. Thus, the root exudate may vary in amount and quality under different conditions.

In any case, it seems evident that, prior to the initial infection, the host greatly affects the fungal associate outside the rootlet by the liberation of material essential to germination (Melin, 1959*a*) and growth of the fungus. The most active growth-promoting constituent of the exudates seems to be the M-factor, although thiamine and occasionally some other B-group vitamins are also essential to the fungi concerned.

The stimulation of the fungus by the exudate appears to influence the fungal production of auxin, which was shown by Slankis to induce a morphogenetic effect on the rootlets of pine. He found that the mycorrhizal mycelia, by releasing this type of hormone, cause an increase in the width of the rootlets and a profuse dichotomous branching similar to that of pine mycorrhiza (Slankis, 1948, 1951, 1958).

The infection of the rootlets by the fungal associate may depend on the availability of energy material in them (Björkman, 1942, 1944), and on the bound M-factor. However, the mechanism of formation of such structures as the Hartig net, and the mycorrhizal sheath, whether attributable to a morphogenetic effect of the inhibitor or of some unknown hormone, still remains to be investigated.

The inhibiting principle produced by the root appears to play a most important part in the establishment of symbiotic relationships. The diffusible inhibitor may, at least in part, determine the susceptibility of the rootlet to infection. It may also, possibly together with a non-diffusible inhibitor (MacDougal & Dufrenoy, 1946), be the agent controlling the extension of the mycorrhizal hyphae in the root.

Thus, the symbiotic relations seem to be largely controlled by growth-promoting as well as growth-inhibiting substances.

It remains to be investigated whether the amounts of active substances in the rootlets are affected by environmental and/or internal factors known or presumed to influence ectotrophic mycorrhiza formation.

Finally, it must be emphasized that much more investigation is necessary before the mechanisms of ectotrophic mycorrhizal formation have been fully explored. As yet only a start has been made in our attempts to understand the relationships between the two symbiotic partners.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Response of mycelial suspension of *Russula xerampelina* Schaeff. ex Fr. to cultured pine roots in maximum nutrient solution. Fungal development occurred almost entirely around the root. From Melin (1954).

Fig. 2. Response of mycelial suspension of *Boletus variegatus* D. to cultured pine roots in a celluloid sack placed on maximum nutrient agar. Mycelial suspension was sieved through wire gauze with holes of 0.01 mm.². Incubation period 7 days. *c.* \times 3.5.

PLATE 2

Fig. 3. Response of mycelial suspension of *Pholiota caperata* Pers. ex Fr. to cultured pine roots on maximum nutrient agar. Mycelial suspension sieved through wire gauze with holes of 0.1 mm.². Incubation period 14 days. \times 2.

Fig. 4. Response of mycelial suspension of *Pholiota caperata* Pers. ex Fr. to cultured pine roots on maximum nutrient agar. Mycelial suspension sieved through wire gauze with holes of *c.* 0.01 mm.². Incubation period 20 days. \times 4.

PLATE 3

Fig. 5. Growth produced by mycelial suspension of *Boletus variegatus* C. in maximum nutrient agar. *Right*, without supplement; *Left*, with the upper parts of the main axis of a cultured pine root (6 months old). Incubation period 10 days. *c.* \times 0.8.

Fig. 6. Response of *Boletus variegatus* C. to secondary rootlets arising from the same pine root as in Pl. 3, fig. 5. Incubation period 7 days. \times 2.5.

PLATE I

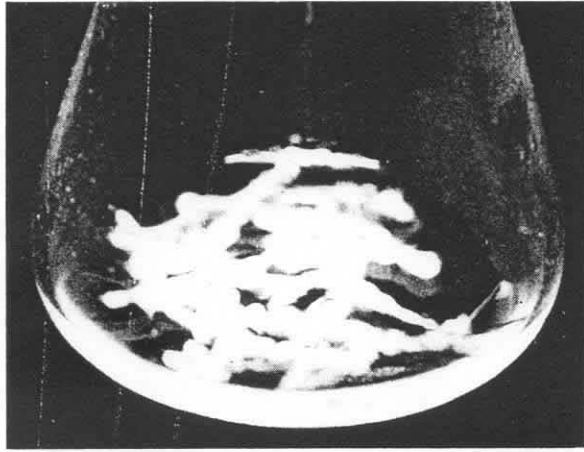


Fig. 1

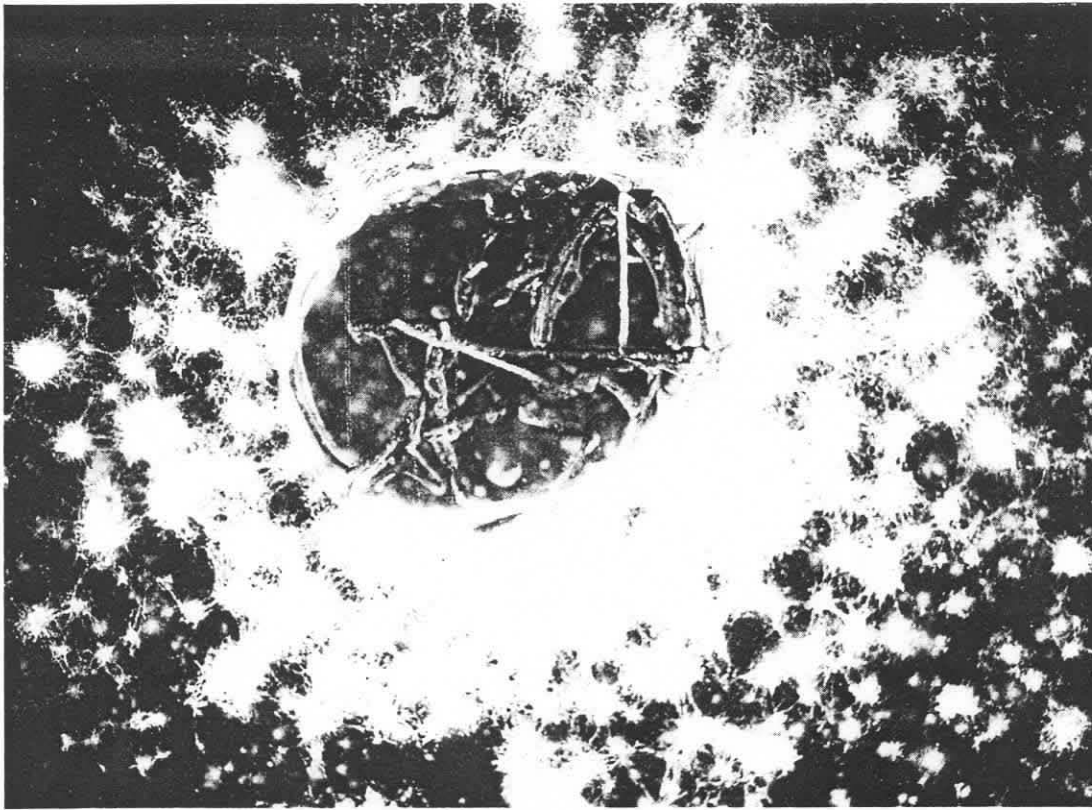


Fig. 2

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PLATE 2

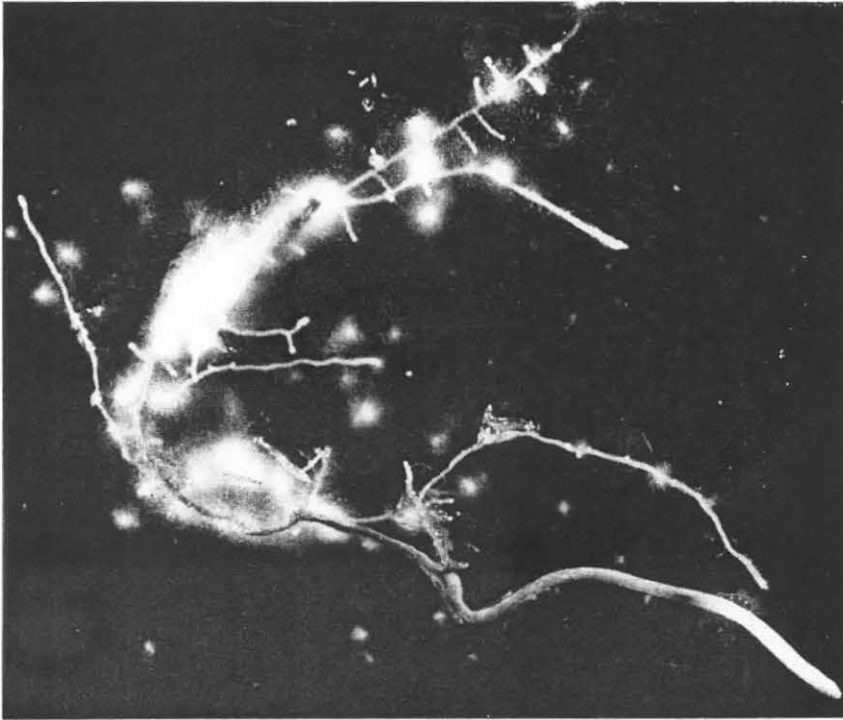


Fig. 3

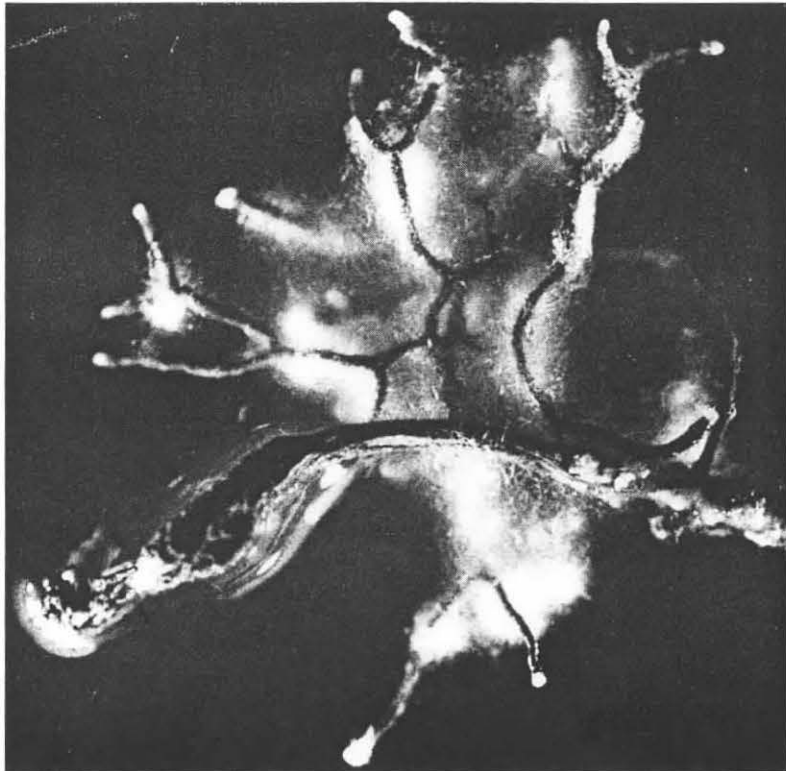


Fig. 4

PLATE 3

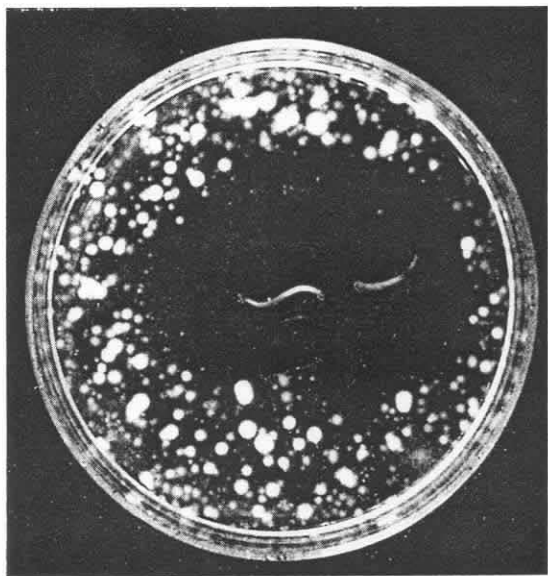


Fig. 5(a)

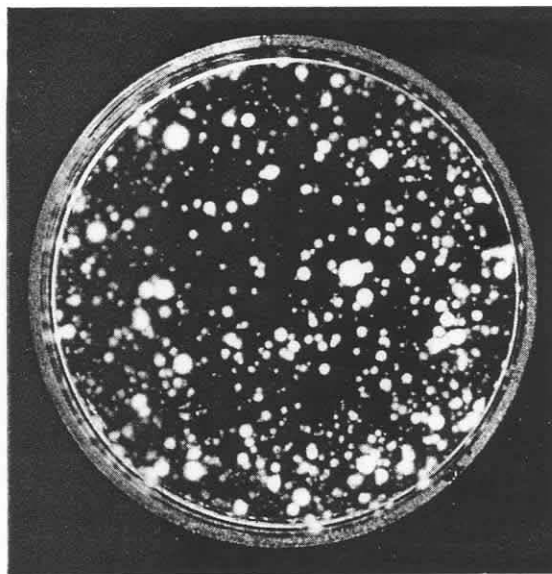


Fig. 5(b)



Fig. 6